Toxicology in Vitro xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

In vitro exposure of tobaccos specific nitrosamines decreases the rat lung phospholipids by enhanced phospholipase A_2 activity

Panneerselvam Vijayaraj [a], Chinnarasu Sivaprakasam [a], Lakshmanaperumal Vishnu Varthini [a], Mary Sarkar [b], Vasanthi Nachiappan [a,*]

[a] *Biomembrane Lab, Department of Biochemistry, School of Life Sciences, Bharathidasan University, Tiruchirappalli, India*
[b] *Biochemistry Department, Indian Institute of Science, Bangalore 560012, India*

ARTICLE INFO

Article history:
Received 13 September 2013
Accepted 5 May 2014
Available online xxxx

Keywords:
Tobacco specific nitrosamines
Pulmonary surfactant
Phospholipids
Dipalmitoylphosphatidylcholine
Phospholipase A_2

ABSTRACT

Tobacco-specific nitrosamines (TSNA) have implications in the pathogenesis of various lung diseases and conditions are prevalent even in non-smokers. N-nitrosonornicotine (NNN) and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are potent pulmonary carcinogens present in tobacco product and are mainly responsible for lung cancer. TSNA reacts with pulmonary surfactants, and alters the surfactant phospholipid. The present study was undertaken to investigate the *in vitro* exposure of rat lung tissue slices to NNK or NNN and to monitor the phospholipid alteration by [^{32}P]orthophosphate labeling. Phospholipid content decreased significantly in the presence of either NNK or NNN with concentration and time dependent manner. Phosphatidylcholine (PC) is the main phospholipid of lung and significant reduction was observed in PC ~61%, followed by phosphatidylglycerol (PG) with 100 μM of NNK, whereas NNN treated tissues showed a reduction in phosphatidylserine (PS) ~60% and PC at 250 μM concentration. The phospholipase A_2 assays and expression studies reveal that both compounds enhanced phospholipid hydrolysis, thereby reducing the phospholipid content. Collectively, our data demonstrated that both NNK and NNN significantly influenced the surfactant phospholipid level by enhanced phospholipase A_2 activity.

1. Introduction

Pulmonary surfactant is a complex system of lipids and proteins that play a vital role in the maintenance of lung function (Obladen, 1992). Phospholipids comprise ~85% of the total surfactant mass (Rau et al., 2003), and lipid composition of the surfactant undergoes various alterations during pathological processes (Matute-Bello et al., 1997; Abraham et al., 2000). Tobacco products are highly toxic that cause a wide range of adverse effects including heart diseases (Tonstad and Johnston, 2006) and pulmonary disorders (Lauren et al., 2008; Robert and Jeanine, 2006; Oulton et al., 1991, 1994). The major tobacco products in cigarette smoke have implications in the pathogenesis of various lung diseases (Hunninghake and Crystal, 1983), and it was observed even in non-smokers who are exposed to tobacco smoke (Willers et al., 1992). However, the mechanisms involved in surfactant alterations are yet to be elucidated.

Tobacco-specific nitrosamines (TSNA), 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) are potent pulmonary carcinogens present in the cigarette (Chen et al., 2008; Hecht, 1998; Stinn et al., 2010). They have been reported by the International Agency for Research on Cancer as human carcinogens (IARC, 2007). Our earlier study in Fischer-344 rats (Nachiappan et al., 1994), revealed that exposure of NNK and NNN increased lipid peroxidation in membrane phospholipids due to excessive generation of reactive oxygen species. NNK alters cell membrane fluidity and surface pressures (Canadas et al., 2011). Recent studies depict that exposure of lipopolysaccharide enhances NNK mediated tumorigenesis in mouse lung (Melkamu et al., 2013). Our laboratory also observed significant alteration in rat lung phospholipid, especially phosphatidylcholine upon exposure to lipopolysaccharide (unpublished data). In addition,

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; EDTA, ethylene diamine tetra acetic acid; EGTA, ethylene glycol tetraacetic acid; FFA, free fatty acid; $LPLA_2$, lysosomal PLA_2; LPC, lysophosphatidylcholine; NNK, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-nitrosonornicotine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; TSNA, tobacco-specific nitrosamines.

* Corresponding author. Tel.: +91 431 2904866; fax: +91 431 2407045.
E-mail address: vasanthibch@gmail.com (V. Nachiappan).

http://dx.doi.org/10.1016/j.tiv.2014.05.001
0887-2333/

lipid homeostasis is a highly regulated process and any alteration in its pathway leads to the creation of a suitable micro-environment for many chronic diseases, including cancer (DeBerardinis et al., 2008). Therefore, it is essential to study the lipid changes upon TSNA exposure. Altered surfactant degradation by secretory phospholipase A_2 ($sPLA_2$), leads to pulmonary dysfunction (Hite et al., 1998; Kitsiouli et al., 2009; Hurley and McCormick, 2008). Lysosomal PLA_2 ($LPLA_2$) plays a significant role in surfactant homeostasis, and during acute lung injury causes inflammatory responses (Abe et al., 2004; Fisher and Dodia, 1997; Fisher et al., 2005). Cigarette smoke reduces phospholipid content (Scott, 2004) by PLA_2 activation (Oulton et al., 1991). We have demonstrated that impaired phospholipid metabolism is due to enhanced phospholipase B activity by NNK (Vijayaraj et al., 2011b) and NNN (Vijayaraj et al., 2011a) in *Saccharomyces cerevisiae*. However, there is no clear evidence for TSNA mediated surfactant degradation in the mammalian system. The present study reveals the effect of *in vitro* exposure of NNN and NNK on the alteration of lung tissue phospholipid is due to enhanced PLA_2 activity resulting in surfactant dysfunction.

2. Materials and methods

[^{32}P]Orthophosphate (5000 μCi/mmol) was obtained from Board of Radiation and Isotope Technology (Mumbai, India). L-α-dipalmitoyl [2-palmitoyl-9,10-^{3}H (N)]-PC (250 μCi/mmol) was obtained from PerkinElmer Life and Analytical Sciences Inc., Massachusetts, USA. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), egg PC, cholesterol and all other lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Bovine serum albumin (BSA), Ethylene diamine tetra acetic acid (EDTA) and Ethylene glycol tetraacetic acid (EGTA) were obtained from Sigma Aldrich Pvt., India. Silica Gel 60_{F254} thin layer Chromatography plates were purchased from Merck (Darmstat, Germany). All chemicals and solvents were purchased from Sigma unless specifically mentioned. NNK, NNN were obtained from Sigma and the purity was confirmed by ESI/MS analysis. Stock solution was prepared in dimethyl sulphoxide (DMSO). Maximum DMSO in the reactions was 0.05% (v/v), a concentration that had no detectable effect on lung phospholipid (data not shown).

2.1. Animals

Male albino rats of Wistar strain, 150–200 g of body weight, were obtained from Central Animal Facility, Indian Institute of Science, Bangalore, India and housed under hygienic and standard environmental conditions at the Central Animal Facility, Bharathidasan University. The rats were fed on a pellet diet (Sree Sai Durga feeds & Foods Pvt., Ltd., Bangalore) and water *ad libitum*. Animals were kept under conventional housing conditions (24 ± 1 °C, 55% humidity, and 12 h day/night cycle). Rats were free from specific respiratory pathogens and were housed in sterile cages and the acclimatization period was at least 7 days before use. All procedures were approved and complied with the standards for the care and use of animal subjects as stated in the guidelines laid down by the Institutional Animal Ethical committee (BDU/IAEC/2010/11 dt. 23/03/2010)., Bharathidasan University.

2.2. Perfusion of lung and sample preparation

The advantage with the lung slice is near identical to in vivo tissues (Sanderson, 2011). Prior to *in vitro* experiments, circulatory and airway cells were removed from the lungs. Briefly, the rats ($n = 6$) were anesthetized with ketamine by intravenous injection (50 mg/kg body weight) and the lungs were perfused with ice-cold saline (0.9% NaCl) via the pulmonary artery and lavaged. Lungs appearing completely white, without pathological alterations were processed for further experiments. The lobes of the perfused lungs were dissected from the main bronchi and lung tissue slices were sectioned into small slices (∼ of 0.7–1 mm size) using tissue chopper. During sectioning, the temperature of the bathing physiological solution was maintained at 4 °C and the slices were kept on nonabsorbent plastic disks for consistent and reproducible weighing of lung mass. Lung tissue slices were then transferred to fresh sterile tube containing physiological solution and kept at 37 °C in a humidified incubator gassed with 5% CO_2/95% air prior to phospholipid labeling. The viability of lung tissue slices were measured by quantification of ATP content in both control and compound treated tissues (De Kanter et al., 2002). All experiments were performed in triplicates and are representative of at least two independent experiments.

2.3. Preparation of subcellular fractions

Perfused lungs were homogenized with homogenization buffer containing 80 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin and 5% sucrose. The homogenate was subjected to centrifugation at 10,000×*g* for 15 min. The supernatant obtained was further centrifuged at 100,000×*g* for 60 min to obtain the cytosolic fraction. The pellet was further washed then centrifuged at 100,000×*g* for 60 min to obtain the microsomal membrane. All the procedures were carried out at 4 °C. Protein concentration was estimated as described by Lowry et al. (1951).

2.4. In vitro [^{32}P]orthophosphate labeling of lung tissue phospholipids

[^{32}P]orthophosphate labeling is a best tool to study the phospholipid metabolism (Dawson, 1954; Sabarirajan et al., 2013). In this present study, lung phospholipids were labeled with [^{32}P]orthophosphate in the presence or absence of NNN or NNK. Alterations were monitored by three different set of experiments (i) lung tissues were incubated with [^{32}P]orthophosphate along with compound, (ii) tissues were incubated with compound followed by phospholipid [^{32}P]orthophosphate labeling (pre-incubation) or (iii) tissue phospholipids were labeled with [^{32}P]orthophosphate followed by incubation with compounds (post-incubation). The alterations in phospholipids were monitored in all the above sets.

Based on the earlier reports (Rioux and Castonguay, 2000; Vijayaraj et al., 2011a,b) we chose a wide range of TSNA (up to 250 μM) to study the phospholipid alteration in lung during *in vitro* conditions. Tissue slices (∼25 mg) were incubated at 37 °C with 5 μCi of [^{32}P]orthophosphate (5000 Ci/mmol) either in the presence or absence of TSNA in phosphate free medium. Various concentrations of TSNA, ranging from 0 to 250 μM were used to study its effect on phospholipids. The effective concentration of TSNA was decided based on the minimum concentration required for maximum phospholipid changes. The maximum incorporation of [^{32}P]orthophosphate was also monitored by incubation of tissue slices in the presence or absence of TSNA at different time points (0–4 h). After incubation, free [^{32}P]orthophosphate was removed with 0.9% NaCl and subjected to lipid extraction (Folch et al., 1957).

2.5. In vitro pre-incubation and post-incubation of lung tissue with TSNA and [^{32}P]orthophosphate labeling

In pre-incubation, tissue slices (25 mg) were incubated at 37 °C with 100 μM NNK or 250 μM NNN for 30 min in phosphate free medium. After treatment, 5 μCi of [^{32}P]orthophosphate was added and incubated for 4 h at 37 °C. Tissues were washed with ice-cold

Please cite this article in press as: Vijayaraj, P., et al. *In vitro* exposure of tobaccos specific nitrosamines decreases the rat lung phospholipids by enhanced phospholipase A_2 activity. Toxicol. in Vitro (2014), http://dx.doi.org/10.1016/j.tiv.2014.05.001